

Regio- and Stereoselective Subterminal Hydroxylations of *n*-Decane by Fungi in a Liquid–Liquid Interface Bioreactor (L–L IBR)

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This article may be the first report to describe the excellent regio- and stereoselective subterminal hydroxylations of *n*-alkane with microorganisms. Approximately 2000 fungal strains were screened for the regioselective hydroxylation of *n*-decane with a solid–liquid interface bioreactor, which is a microbial transformation device on an interface between an agar plate and an organic phase (*n*-decane). Although *Beauveria bassiana* ATCC 7159, a typical fungus having a strong and versatile hydroxylating ability, mainly produced a mixture of 2-, 3-, 4-, and 5-decanone regioisomers into a *n*-decane layer, *Monilliela* sp. NAP 00702 and *Rhizopus oryzae* R-38-8 typically accumulated 4- and 5-decanols into the *n*-decane layer, respectively. Although a small amount of 4- and 5-decanones were also produced, the regioselectivity at C-4 and C-5 positions of *n*-decane reached 99 and 90%, respectively. Furthermore, the hydroxylation of *n*-decane with *Monilliela* sp. NAP 00702 stereoselectively proceeded to afford almost 100% ee of (−)-4-decanol.

Fungi have various cytochrome P-450 systems, versatile hydroxylating enzyme systems, to afford many useful hydroxylated products. For examples, plant pathogenic *Cunninghamella*, *Curvularia*, and *Botrytis* have been applied to various hydroxylations of natural or artificial substances[1–3] besides some harmless fungi such as *Aspergillus*, *Absidia*, *Mucor*, and *Rhizopus*.[4–7] Concerning the hydroxylation of methylene groups, microbial hydroxylation preferentially occurs at activated positions, such as benzyl and aryl sites in general.[8,9] Hydroxylation of non-activated carbons is very difficult by either chemical or enzymatic procedures.

However, it is well known that *n*-alkanes, consisting of non-activated methyl and methylene groups, are degraded via terminal and/or subterminal hydroxylation by various microorganisms.[10] As for the hydroxylation of *n*-alkanes by bacteria, alkane hydroxylase in *Pseudomonas putida*, which consists of integral membrane monooxygenase, rubredoxin, and rubredoxin reductase, catalyzes the terminal hydroxylation of *n*-alkanes.[11,12] While *Brevibacterium erythrogenes*,[13] *Micrococcus cerificans*,[14] *Nocardia salmonicolor*,[15] and *Corynebacterium* sp.[16,17] also hydroxylate a terminal methyl group of *n*-alkanes, *Arthrobacter* sp. catalyzes the subterminal hydroxylation of *n*-hexadecane to afford 2-, 3-, and 4-hexadecanols.[18] Recently, it has been reported that an engineered cytochrome P450BM-3 catalyzes both terminal and subterminal hydroxylations of *n*-alkanes.[19]

On the other hand, some yeasts also catalyze monoterminal, diterminal, and/or subterminal hydroxylations of *n*-alkanes. For example, *Candida intermedia*,[20] *C. parasilosis*,[21] *C. guilliermondii*,[22] and *Lodderomyces elongisporus*[23] hydroxylate a terminal methyl group of *n*-alkanes, while *Candida lipolytica* hydroxylates both terminal and C-2 methylene groups of *n*-alkanes.[24] Concerning fungal hydroxylations of *n*-alkanes, while *Cladosporium resinae* hydroxylates a terminal methyl group,[25] *Cunninghamella blakesleeana* hydroxylates both terminal methyl and subterminal methylene groups of *n*-alkanes.[26,27]

As for the subterminal hydroxylation of *n*-alkanes, there is no report of successful highly regio- and stereoselective hydroxylations of *n*-alkanes. In this article, we demonstrate the first report of regio- and stereoselective hydroxylation of *n*-decane with fungi in a liquid–liquid interface bioreactor (L–L IBR), which is a unique bioconversion system using fungal cells growing on an interface between a liquid medium and a hydrophobic organic solvent.[28,29] In this system, fungal cells are spontaneously immobilized on the liquid–liquid interface by the aid of ballooned microsphere particles to differentiate naturally. The cells efficiently convert hydrophobic substrates dissolved in the organic solvent to hydrophobic products. The accumulation of the product in the organic solvent reaches a higher level compared to emulsion and organic–aqueous two-liquid-phase systems, or a solid–liquid interface bioreactor (S–L IBR).[30–34] Using the L–L IBR system, *Monilliela* sp. NAP 00702 and *Rhizopus oryzae* R-38-8 catalyzed regioselective hydroxylation of *n*-decane to afford 4- and 5-decanol in high yields, respectively. Furthermore, the regioselective subterminal hydroxylation by *Monilliela* sp. NAP 00702 stereoselectively proceeded to afford almost 100% ee of (−)-4-decanol. On the other hand, while 2- and 3-decanol were produced by *Colletotrichum linicolum* and *Aspergillus tunetana* with moderate regioselectivities, *Trichoderma* sp. afforded 1-decanol regioselectively (Figure 1).

Experimental

Screening of *n*-Decane-Hydroxylating Fungi. All fungi tested were stocked in the Bioresource Laboratories of Mercian Corporation, containing type culture strains from ATCC (American Type Culture Collection), NRRL (Agricultural Research Service

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Culture Collection), and NBRC (NITE Biological Resource Center; former IFO). All strains were stocked on modified Sabouraud agar plates consisting of 40.0 g of glucose, 10.0 g of Bacto peptone, 5 mg of $FeSO_4 \cdot 7H_2O$, 20 mg of $MnSO_4 \cdot 5H_2O$, 10 mg of $CaCl_2$, and 15.0 g of agar in 1.0 L of deionized water (pH 6.0) at 4 °C. Approximately 2000 strains were screened with the S–L IBR (vessel, glass vial; volume, 50 mL; diameter, 3 cm) consisting of the modified Sabouraud agar plate (volume, 10 mL; surface area, 7.1 cm^2) and *n*-decane (1.5 mL). Three pieces (approximately 2 mm × 2 mm) of each fungal mat were inoculated on the surface of the agar plate with a long toothpick, and precultivation was done at 25 °C by allowing the plate to stand for 3 days. After the precultivation, 1.5 mL of *n*-decane was added onto the surface of a fungal mat. The stationary incubation was continued at 25 °C for 7 days. After the incubation, the *n*-decane layer in the vessel was directly analyzed by gas chromatography. The column (0.25 mm i.d. × 60 m) contained SUPELCOWAX-10 (Supelco Co., Ltd., Bellefonte, PA). The column temperature was raised from 80 to 110 °C (2 °C min^{-1}), held at 110 °C for 5 min, raised from 110 to 120 °C (2 °C min^{-1}), held at 120 °C for 5 min, raised from 120 to 150 °C (6 °C min^{-1}), and held at 150 °C for 10 min. The injector and detector temperatures were 155 and 160 °C, respectively. The carrier gas and split ratio were He (20 s min^{-1}) and 1:100, respectively. The retention times of 5-, 4-, 3-, and 2-decanones and 5-, 4-, 3-, 2-, and 1-decanols were 21.22, 21.36, 23.47, 25.66, 31.63, 31.79, 32.79, 34.37, and 42.54 min, respectively. The products were identified by GC-MS (Turbo Mass Gold, Perkin-Elmer., Inc., Waltham, MA) by comparison with authentic samples and an attached database. The GC-MS measurements were acquired under the following conditions; ionization was by electron impact, ionization voltage was 0.6 eV, ion source temperature was 50 °C. GC conditions were identical for both qualitative and quantitative analysis.

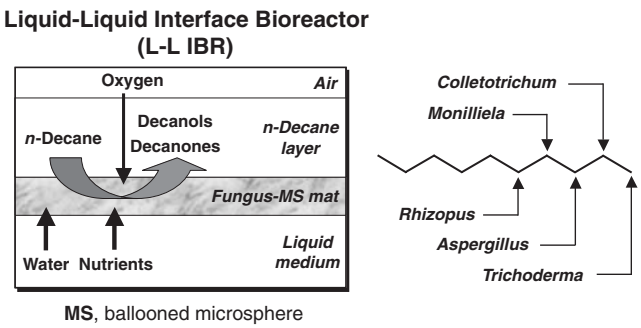
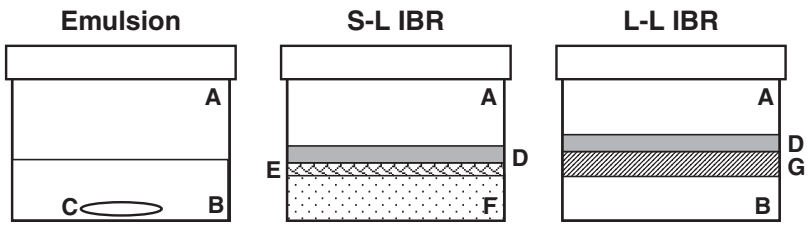


Figure 1. Principle of liquid–liquid interface bioreactor (L–L IBR) and its application to regioselective hydroxylations of *n*-decane with fungi. A fungus-microsphere mat located on an interface between a liquid medium and *n*-decane regioselectively hydroxylates *n*-decane.

Comparison of *n*-Decane-Hydroxylating Abilities of *Monilliela* sp. NAP 00702 between Emulsion and L–L IBR Systems. The F1 liquid medium consisted of 20.0 g of potato starch, 10.0 g of glucose, 20.0 g of soy protein (Soypro™, Inui Co., Ltd., Osaka), 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$ in 1.0 L of deionized water (pH 6.0) was used for seed cultivation. As for the emulsion system (Figure 2), 4 mL of a 3-day seed broth, which was prepared by shaken cultivation (25 °C, 220 rpm), was inoculated to 50 mL of the modified Sabouraud liquid medium. After precultivation at 25 °C with agitation (300 rpm) for 3 days, 20 mL of *n*-decane was added to the broth and incubation was continued at 25 °C with agitation (300 rpm) for 9 days. As for the L–L IBR system (Figure 2), 4 mL of a 3-day broth was inoculated to the modified Sabouraud liquid medium containing 1.0 g of ballooned polyacrylonitrile microsphere (MFL-80GCA, $CaCO_3$-coated type; mean diameter, 20 μm; density, 0.2; Matsumoto Yushi-Seiyaku, Co., Ltd., Osaka). After 3-day stationary precultivation at 25 °C, 20 mL of *n*-decane was added onto the surface of a fungus-microsphere mat and incubation was continued at 25 °C by allowing the reactor to stand for 9 days. Products in the *n*-decane layer were directly determined by gas-chromatography in the above-mentioned manner. The enantiomeric excess of 4-decanol produced was also directly determined by gas-chromatography. The column (0.25 mm i.d. × 30 m) contained β-DEX™ 325 (Supelco Co., Ltd.). The column, injector, and detector temperatures were 160, 165, and 170 °C, respectively. The carrier gas and split ratio were He (20 s min^{-1}) and 1:100, respectively. The retention times of (−)- and (+)-4-decanol were 17.61 and 17.89 min, respectively.

Comparison of *n*-Decane-Hydroxylating Abilities of *Rhizopus oryzae* R-38-8 among Emulsion, S–L IBR, and L–L IBR Systems. Schematic diagrams of emulsion, S–L IBR, and L–L IBR systems are shown in Figure 2. As for the emulsion system, 4 mL of a homogenate of a fungal mat formed on the agar plate for 7 days was added to 60 mL of the modified Sabouraud liquid medium. After 3-day precultivation at 25 °C with agitation (300 rpm), 20 mL of *n*-decane was added to the broth and cultivation was continued for 5 days. Concerning the S–L IBR system, 2 mL of the homogenate was inoculated onto the surface of the modified Sabouraud agar plate (volume, 60 mL; surface area, 38.5 cm^2). After 3-day stationary precultivation at 25 °C, 20 mL of *n*-decane was added onto the surface of a fungal mat and cultivation was continued for 5 days. As for the L–L IBR system, 4 mL of the homogenate was added to 60 mL of the liquid medium containing 1.2 g of ballooned polyacrylonitrile microsphere (MFL-80GTA, talc-coated type; mean diameter, 20 μm; density, 0.2; Matsumoto Yushi-Seiyaku, Co., Ltd., Osaka). After 3-day sta-



A, Deep Petri dish (height, 5 cm; diameter, 7 cm); **B**, liquid medium; **C**, magnet; **D**, *n*-decane layer; **E**, fungal mat; **F**, agar plate; **G**, fungus-microsphere mat

Figure 2. Schematic diagrams of emulsion, S–L IBR, and L–L IBR systems. All systems are shown as vertical sections.

Table 1. Screening of Fungi Catalyzing Regioselective Hydroxylation of *n*-Decane

Strain	Product concentration/mg mL^{-1}								
	5K	**4K**	**3K**	**2K**	**5A**	**4A**	**3A**	**2A**	**1A**
Beauveria bassiana ATCC 7159	2.60	1.02	1.98	3.39	0.30	0.20	0.33	0.32	nd
Trichoderma sp. f 16062	nd[b)]	nd	nd	nd	nd	tr[a)]	tr	tr	1.78
Colletotrichum linicolum G-4	nd	nd	0.03	0.08	0.03	0.11	1.16	2.52	nd
Aspergillus tunetana var. *pallidas* A-124	tr	0.09	0.26	0.06	0.12	0.41	1.60	0.11	0.17
Monilliela sp. NAP 00702	nd	0.22	tr	tr	0.05	1.44	0.04	nd	nd
Rhizopus oryzae R-38-8	2.88	tr	nd	nd	7.65	0.83	0.11	0.07	0.18

a) tr, Trace amount. b) nd, not detected. **5K**, 5-decanone; **4K**, 4-decanone; **3K**, 3-decanone; **2K**, 2-decanone; **5A**, 5-decanol; **4A**, 4-decanol; **3A**, 3-decanol; **2A**, 2-decanol; and **1A**, 1-decanol. After 3-days precultivation, 1.5 mL of *n*-decane was added onto a fungal mat (surface area, 7.1 cm^2) and incubation was statically done at 35 °C for 7 days.

tionary precultivation at 25 °C, 20 mL of *n*-decane was added onto the surface of a fungus-microsphere mat. Incubation was continued at 25 °C by allowing the reactor to stand for 5 days. Products in the *n*-decane layer were directly analyzed by gas-chromatography in the above-mentioned manner.

Results and Discussion

Since the early nineteen-sixties, numerous reports of assimilation and oxidation of *n*-alkanes by microorganisms have been published.[10,35] Terminal and subterminal hydroxylations are initial steps of degradation of *n*-alkanes with the aid of alkane hydroxylase or cytochrome P-450 systems. While the terminal hydroxylation by microorganisms is highly regioselective in general, the regioselectivity of microbial subterminal hydroxylation is very low. Moreover, microbial subterminal hydroxylation of fatty acids, linear-chain compounds similar to *n*-alkanes, also proceeds non-regioselectively.[36–38] Thus, recognition of the difference of hydroxylated positions in enzymatic and chemical reactions is very difficult. Toward the above-mentioned traditional information, we tried to discover regio- and stereoselective subterminal hydroxylation of *n*-alkanes with fungi.

Screening of Microorganisms Catalyzing Regioselective Subterminal Hydroxylation of *n*-Decane. First, approximately 2000 fungal strains were applied to the S–L IBR consisting of an agar plate and *n*-decane layer. The bioreactor is an efficient and conventional device for the microbial transformation of water-insoluble substrates because of the excellent substrate- and/or product-toxicity alleviation effect and the solubilization of the substrates.[30–34] The organic phase serves as a reservoir and a solvent of toxic substrates and products. In the case of this study, *n*-decane was used as the organic phase and the substrate. As shown in Table 1, *Beauveria bassiana* ATCC 7159 having a strong and versatile hydroxylating activity[39,40] mainly afforded a mixture of 2-, 3-, 4-, and 5-decanones. It was confirmed that the fragmentation patterns of each product was identical with corresponding authentic sample and an attached database by the aid of GC-MS. However, *Monilliela* sp. NAP 00702 and *Rhizopus oryzae* R-38-8 regioselectively hydroxylated *n*-decane to afford mainly 4- and 5-decanol, respectively. The regioselectivities (alcohol plus ketone per sum of all products) of *Monilliela* sp. NAP 00702 and *R. oryzae* R-38-3 reached to 99 and 90%, respectively. On the other hand, *Colletotrichum linicolum* G-4 and *Aspergillus tunetana* var. *pallidas* A-124 hydroxylated C-2 and C-3 positions of *n*-decane at moderate regioselectivity. *Trichoderma* sp. f16062 regioselectively catalyzed terminal hydroxylation of *n*-decane to afford 1-decanol. As for the production of 5-decanol, many *Rhizopus* strains also exhibited superior regioselectivity for the subterminal hydroxylation of *n*-decane as shown in Table 2. Although oxidation of alkanols to alkanones proceeded in part, the accumulation of all alkanols reached over 1 g L^{-1} in the S–L IBR because of its effective toxicity alleviation effect. Especially, *R. oryzae* R-38-8 accumulated 7.65 mg of 5-decanol in 1 mL of *n*-decane layer in spite of the high biotoxicity of the alkanol.[41]

Comparison of *n*-Decane-Hydroxylating Abilities of *Monilliela* sp. NAP 00702 between Emulsion and L–L IBR Systems. It was reported that the L–L IBR enabled the higher accumulation of hydrophobic products such as 2-ethyl-1-hexanol[28] and (*S*)-benzoin[29] compared with emulsion, organic–aqueous two-liquid-phase, and S–L IBR systems. Thus, the regioselective subterminal hydroxylation of *n*-decane with *Monilliela* sp. NAP 00702 was applied to the emulsion and the L–L IBR systems. As shown in Figure 3A, although initial hydroxylation rate in the emulsion system was higher than that in the L–L IBR system, 4-decanol and 4-decanone were degraded after the 4th day in the emulsion system. It was assumed that 4-decanol was degraded via Baeyer–Villiger oxidation of 4-decanone in the emulsion system likewise in some other microorganisms.[42,43] Thus, the *n*-decane layer in the emulsion system with vigorous agitation might not sufficiently function as a reservoir of 4-decanol in the same way as a dispersed organic–aqueous two-liquid-phase system.[44,45]

On the other hand, the accumulation of 4-decanol continued after the 4th day in the L–L IBR system. It is easily assumed that a stationary *n*-decane layer acts as a reservoir of 4-decanol and 4-decanone to alleviate the degradation of these products.

Furthermore, it was observed that the subterminal hydroxylation of *n*-decane by *Monilliela* sp. NAP 00702 stereoselectively proceeded to afford (−)-isomer. The enantiomeric excess of (−)-4-decanol was almost 100% as shown in Figures 3B and 3C. As for the stereoselectivity of subterminal hydroxylation of *n*-alkanes, it was reported that an engineered cytochrome P450BM-3 (9-10A-A328V) afforded 55% ee of (*R*)-2-decanol.[19] It was also reported that the stereoselectivities of subterminal hydroxylations of some carboxylic acids and *n*-alkylbenzenes with other P-450 systems were high, however, the regioselectivities of the hydroxylations were very low.[46–48] Thus, it is concluded that the hydroxylation of *n*-decane with

Table 2. Product Profiles of Hydroxylation of *n*-Decane with *Rhizopus* spp.

Strain	Product concentration/mg mL^{-1}									**5K + 5A**
	5K	**4K**	**3K**	**2K**	**5A**	**4A**	**3A**	**2A**	**1A**	/%
R. oryzae R-38-8	2.88	tr[a)]	nd[b)]	nd	7.65	0.83	0.11	0.07	0.18	89.9
R. oryzae R-25-3	2.17	tr	nd	nd	7.25	0.73	0.14	0.11	0.08	89.9
Rhizopus sp. IFO 4789	1.78	tr	nd	nd	6.64	0.79	0.10	0.06	0.08	89.1
R. oryzae R-35-5	1.83	tr	nd	nd	6.41	0.80	0.09	0.06	0.08	88.9
Rhizopus sp. IFO 4723	1.52	tr	nd	nd	5.78	0.71	0.08	0.05	0.07	88.9
R. oryzae R-28-5	1.32	tr	nd	nd	5.65	0.71	0.08	0.05	0.06	88.6
R. oryzae R-32-5	1.54	tr	nd	nd	5.63	0.71	0.08	0.05	0.07	88.7
R. oryzae R-27-8	3.20	tr	nd	nd	5.27	0.64	0.08	0.06	0.29	88.8
R. oryzae IFO 4728	1.16	tr	nd	nd	5.11	0.62	0.07	0.05	0.05	87.6
R. oryzae R-12-10	2.54	tr	nd	nd	4.38	0.57	0.06	0.03	nd	91.3
R. oryzae IAM 6002	3.49	tr	tr	tr	3.98	0.60	0.12	0.06	0.30	87.4
R. delemar IFO 4786	2.17	tr	nd	nd	3.73	0.44	0.05	0.03	0.19	89.3
R. delemar IFO 4806	1.85	tr	nd	nd	2.96	0.44	0.07	0.04	0.12	87.8
R. shanghaiensis IFO 4888	1.68	tr	nd	nd	2.82	0.44	0.08	0.04	0.10	87.2
R. oryzae R-43-1	0.90	tr	nd	nd	2.65	0.34	0.03	tr	0.05	89.4
R. oryzae R-41-8	0.78	tr	nd	nd	2.49	0.35	0.03	tr	0.04	88.6
R. oryzae IFO 4716	0.69	tr	nd	nd	2.49	0.33	tr	nd	0.03	89.8
R. oryzae IAM 6065	0.38	tr	nd	nd	2.00	0.28	0.07	0.06	nd	85.3

a) tr, Trace amount. b) nd, not detected. **5K**, 5-decanone; **4K**, 4-decanone; **3K**, 3-decanone; **2K**, 2-decanone; **5A**, 5-decanol; **4A**, 4-decanol; **3A**, 3-decanol; **2A**, 2-decanol; and **1A**, 1-decanol. Reaction conditions were the same as in the footnote to Table 1.

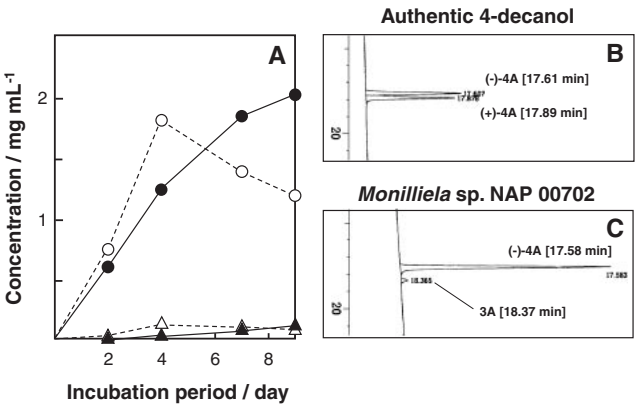


Figure 3. Time course of hydroxylation of *n*-decane with *Moniliella* sp. NAP 00702 and chiral gas chromatogram of 4-decanol (**4A**) produced. (A) Time course of hydroxylation. Open circles, **4A** produced in emulsion system; closed circles, **4A** produced in L–L IBR system; open triangle, 4-decanone (**4K**) produced in emulsion system; closed triangle **4K** produced in L–L IBR system. (B) Chiral gas chromatogram of authentic 4-decanol. (C) Chiral gas chromatogram of 4-decanol produced by *Monilliela* sp. NAP 00702. A small amount of 3-decanol (**3A**) was also produced.

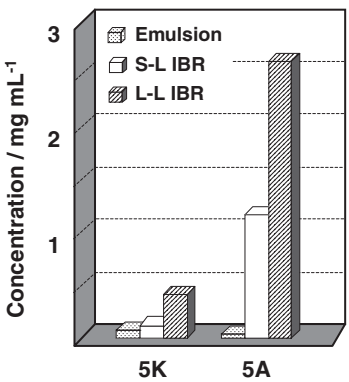


Figure 4. Comparison of 5-decanol and 5-decanone production with *R. oryzae* R-38-8 among emulsion, S–L IBR, and L–L IBR systems. **5K**, 5-decanone; **5A**, 5-decanol. While the emulsion system was conducted at 25 °C with agitation (300 rpm) for 5 days, the S–L and L–L IBR systems were statically conducted at 25 °C.

Monilliela sp. NAP 00702 may be the first report for the regio- and stereoselective subterminal hydroxylation of *n*-alkanes with microorganisms.

Comparison of *n*-Decane-Hydroxylating Abilities of *Rhizopus oryzae* R-38-8 among Emulsion, S–L IBR, and L–L IBR Systems. Efficient production of hydroxylation product in the L–L IBR system was also estimated for the production of 5-decanol with *R. oryzae* R-38-8. As shown in Figure 4, the accumulation of 5-decanol and 5-decanone in the L–L IBR system was significantly higher than those in the emulsion and the S–L IBR systems. In the emulsion system, the accumulations of 5-decanol and 5-decanone were very low level because *R. oryzae* R-38-8 formed a large aggregate in the broth. It is well known that the control of fungal morphology is a very important factor for fermentation and microbial transformation.[49,50] In conclusion, the easy morphology control of *R. oryzae* R-38-8, the higher contents of the substrate and oxygen in the organic phase, and the excellent toxicity alleviation effect in the L–L IBR system enables the higher production of 5-decanol.

In order to gain a series of optically active decanol isomers in higher yields, both the deletion of secondary alcohol dehydrogenase and the selection of other fungi having excellent

regio- and stereoselective C-2 and C-3 hydroxylation activities must be achieved. Moreover, it is expected that a large scale L–L IBR system will be constructed. We have continued various trials for resolving the above-mentioned tasks.

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